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Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron

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Phenanthroline, a strong iron chelator, prevents both the formation of DNA single-strand breaks and the killing of mouse cells produced by H_2O_2. These results, taken together with our previous findings, indicate that the DNA damage is produced by hydroxyl radicals formed when H_2O_2 reacts with chromatin-bound Fe^{2+} and that this damage is responsible for the killing effect.

The active oxygen species H_2O_2, superoxide anion (O_2^-) and hydroxyl radical ($OH^\cdot$) have been implicated as a cause of cellular death (McCord, 1974; Fridovich, 1975; Hoffmann & Meneghini, 1979), mutagenesis and carcinogenesis (Moody & Hassan, 1982; Borek & Troll, 1983), aging (Tolmasoff *et al.*, 1980), arthritis (McCord, 1974), ischaemic damage (Siesjo *et al.*, 1980) and emphysema (Dooley & Pryor, 1982). We have been interested in the mechanism of cellular death caused by H_2O_2 because (i) it seems to involve DNA as a target (Hoffmann & Meneghini, 1979) and therefore may provide a key to its action in mutagenesis, carcinogenesis and aging, and (ii) H_2O_2 is likely to be related to other active oxygen species by cellular reactions similar to those occurring *in vitro* (see Gutteridge *et al.*, 1982). Cellular death by H_2O_2 may therefore constitute a good model for understanding the deleterious action of active oxygen species in cellular systems.

We have previously found that H_2O_2 causes DNA single-strand breaks in human fibroblasts (Hoffmann & Meneghini, 1979). Because H_2O_2 did not attack purified DNA, we concluded that an intracellular species was mediating the production of lesions. One possibility is that the mediator is intracellular iron (Meneghini & Hoffmann, 1980), which might react with H_2O_2 by a Fenton (1893) type of reaction, producing the very reactive hydroxyl radical:

$$H_2O_2 + Fe^{2+} \rightarrow OH^\cdot + OH^- + Fe^{3+}$$

In this case the hydroxyl radical would be the ultimate species to attack DNA. We have now corroborated this hypothesis by using the powerful iron chelator 1,10-phenanthroline. This compound proved to be very effective in penetrating the cells and inhibiting the DNA-damaging action of H_2O_2. Moreover, a concomitant protection of the cells against the lethal action of H_2O_2 was observed, which strongly suggests DNA as the target for this lethal effect.

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Materials and methods

Mouse cells (3T3) were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal-calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml in Pyrex Petri dishes at 37°C in a humidified air/CO_2 (19:1) atmosphere. Radioactive labelling, treatment with H_2O_2 and sedimentation in alkaline sucrose gradients were carried out as described previously (Meneghini, 1976; Hoffmann & Meneghini, 1979). Briefly, the treatment of the cells was in phosphate-buffered saline (0.137 M-NaCl/2.7 mM-KCl/8 mM-Na_2HPO_4/1.5 mM-KH_2PO_4) with H_2O_2 and 1,10-phenanthroline at the indicated concentrations. Incubation was for 30 min at 37°C in the dark. Assay for survivors was carried out as described previously (Hoffmann & Meneghini, 1979). Survival was scored as the ability of a single cell to form a colony of at least 50 cells.

Results

When mouse 3T3 cells are incubated with 120 μM-H_2O_2, single-strand breaks appear in their DNA (Fig. 1*a*). This is similar to what has previously been observed for human fibroblasts (Hoffmann & Meneghini, 1979), although at a

similar H_2O_2 concentration more DNA single-strand breaks are formed in human fibroblasts than in mouse fibroblasts. If the cells are incubated in the presence of 120 μM-H_2O_2 and 100 μM-1,10-phenanthroline, their DNA is completely protected from single-strand-break formation (Fig. 1*b*). Incubation with 1,10-phenanthroline alone yields the same DNA-sedimentation profile as the control (Fig. 1*c*). Essentially identical results (not shown) are obtained with another iron chelator, 2,2'-bipyridine ('αα'-dipyridyl').

Fig. 2 shows the ability of individual 3T3 cells to form colonies as a function of H_2O_2 concentration. The curve presents an initial shoulder followed by an exponential section. The H_2O_2 concentration that produces an average of one lethal event per cell is 15 μM. If the cells are incubated simultaneously with 100 μM-1,10-phenanthroline and 15 or 36 μM-H_2O_2, they become completely resistant toward the latter. 1,10-Phenanthroline alone has no effect on survival. The protection it lends to the cells is complete even at 35 μM-H_2O_2, a concentration that, in the absence of 1,10-phenanthroline, kills 95% of the cells.

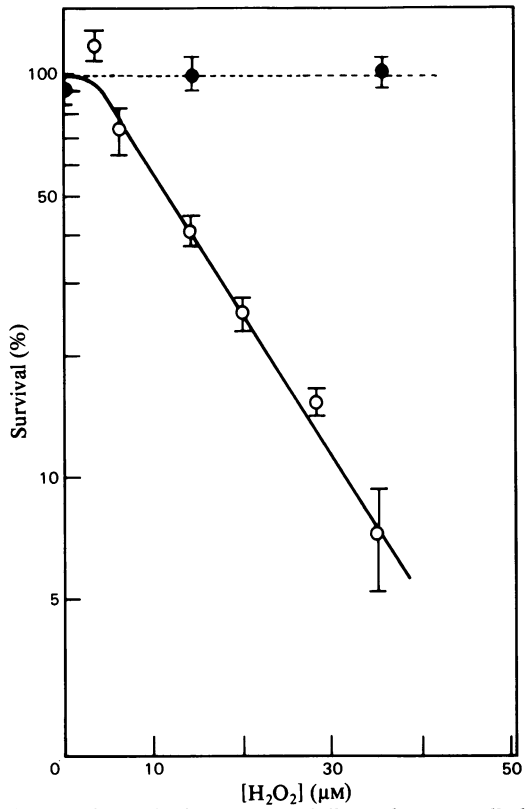

Fig. 2. *Phenanthroline prevents killing of mouse cells by H_2O_2*
Cells were treated with the indicated concentrations of H_2O_2 in the absence (○) or presence (●) of 100 μM-1,10-phenanthroline. Bars indicate deviations from the mean in two separate determinations.

Discussion

The powerful iron chelator 1,10-phenanthroline completely protected intracellular DNA from single-strand-break formation produced by H_2O_2. The simplest explanation for this effect is that this compound enters the cells and removes iron from the chromatin, since this cellular structure is firmly bound to the species that mediated the DNA-

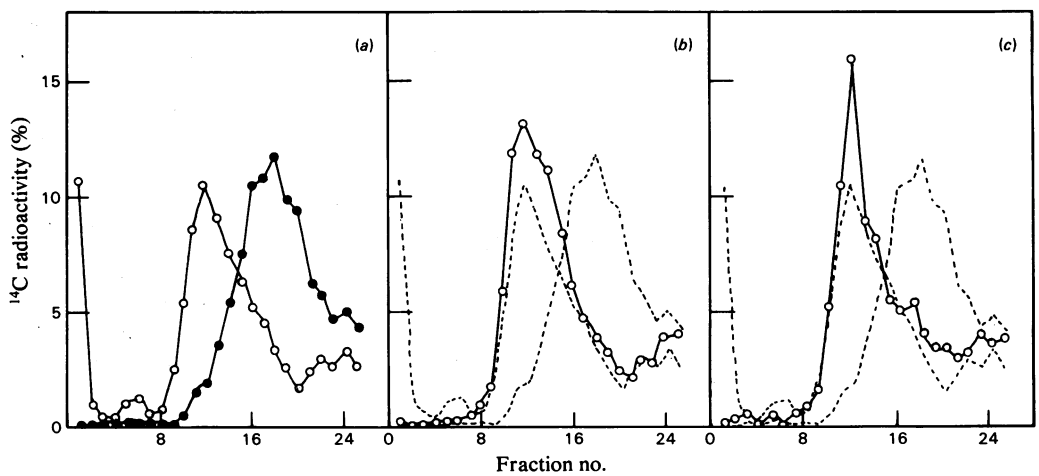

Fig. 1. *Phenanthroline prevents single-strand breaks formation in DNA of mouse cells by H_2O_2*
Cellular DNA was prelabelled by incubating cells for 36h in medium containing 0.1 μCi of [^{14}C]thymidine/ml. In (*a*), cells were treated with phosphate-buffered saline (○) or 120 μM-H_2O_2 (●). The resulting DNA-sedimentation profiles in alkaline sucrose gradients (sedimentation from right to left) are depicted by broken lines (----) in (*b*) and (*c*), which also show the results that were obtained when cells were treated with 120 μM-H_2O_2 plus 100 μM-1,10-phenanthroline (*b*) and 100 μM-1,10-phenanthroline alone (*c*).

damaging action of H_2O_2 (Meneghini & Hoffmann, 1980). At these sites, iron reacts with H_2O_2 according to the Fenton-like reaction described in the introduction, and the $OH^{\cdot}$ radical thus formed is the species that attacks DNA.

The complexation of iron by 1,10-phenanthroline brings about an increase in the redox potential of the metal such that it can no longer participate in the Fenton reaction (Burgess & Prince, 1965). At the same time as it protects DNA from H_2O_2-induced lesions, 1,10-phenanthroline also protects the cell from potentially lethal damage produced by H_2O_2. Similar results were obtained with another iron chelator, 2,2′-bipyridine. These findings clearly demonstrate that intracellular iron is the mediator of both the DNA-damaging action and the killing activity of H_2O_2. They also indicate that the DNA lesions may be responsible for the killing effect of H_2O_2, although the possibility cannot be ruled out that the damage to other cellular structures by H_2O_2 is also mediated by iron and contributes to killing. Whether superoxide anion is involved in the formation of hydroxyl radical by an iron-catalysed Haber–Weiss reaction (Halliwell, 1982) requires further investigation.

These results corroborate the previous findings that DNA single-strand-breaks production by H_2O_2 in mammalian nuclei is mediated by a EDTA-inactivated species (Meneghini & Hoffmann, 1980). They are also in agreement with the finding of Repine *et al.* (1981) that growing *Staphylococcus aureus* in media containing increasing concentrations of iron increased their content of iron and dramatically enhanced their subsequent susceptibility to killing by H_2O_2.

The recent finding that H_2O_2 is the species responsible for the lethal injury to human fibroblasts produced by phagocytic cells at the site of inflammation (Simon *et al.*, 1981), plus our observations reported here, seem to shed light on the mechanism of killing by phagocytic cells.

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